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13. ABSTRACT ***(Maximum 200 Words)***

A significant proportion of human breast cancers overexpress ErbB2, a member of the receptor tyrosine kinase gene family that also includes the epidermal growth factor receptor (EGFR). Overexpression of ErbB2 correlates with increased metastatic potential and poorer prognosis. Agents that antagonize the activity of ErbB family members have obvious clinical implications. We have previously demonstrated that decorin is a novel ligand for the EGFR. This interaction triggers a signaling cascade that leads to activation of MAP kinase and ultimately to growth suppression. In the preliminary data that support the basis of this proposal, we discovered that decorin causes a functional inactivation of the oncogenic ErbB2 protein in mammary carcinoma cells overexpressing ErbB2. This leads to growth inhibition and cytodifferentiation of mammary tumor cells and a concurrent suppression of their tumorigenic potential in vivo. We demonstrate that transient transgene expression of decorin causes a significant growth inhibition of tumor xenografts. These cytostatic effects correlated with a reduced proliferative index and an attenuation of EGFR phosphorylation *in vivo*. Thus, decorin gene therapy helps in retarding the growth of human tumors in immunocompromised animals and could represent a new independent or adjunctive therapeutic modality against cancer.

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INTRODUCTION

A significant proportion of human breast cancers overexpress ErbB2, a member of the receptor tyrosine kinase gene family that also includes the epidermal growth factor receptor (EGFR). Overexpression of ErbB2 correlates with increased metastatic potential and poorer prognosis. Agents that antagonize the activity of ErbB family members have obvious clinical implications. We have previously demonstrated that decorin is a novel ligand for the EGFR. This interaction triggers a signaling cascade that leads to activation of MAP kinase and ultimately to growth suppression. In the preliminary data that support the basis of this proposal, we discovered that decorin causes a functional inactivation of the oncogenic ErbB2 protein in mammary carcinoma cells overexpressing ErbB2. This leads to growth inhibition and cytodifferentiation of mammary tumor cells and a concurrent suppression of their tumorigenic potential in vivo. We hypothesize that expression of decorin by breast carcinoma cells in an *in vivo* tumor model will result in inhibition of tunmor growth. We further hypothesize that exogenous administration of the human decorin transgene via a viral or liposomal transfer vector will lead to growth slowdown and/or growth inhibition of already established xenograft tumors of human breast carcinomas.

BODY

To date we have made significant progress in some areas of our proposal, and additionally are in the process of developing improved strategies to overcome several current shortcomings and limitations of our AAV-based vector system.

AAV2 decorin vector

The AAV2 vector has been proving problematic for efficient gene transfer both *in vitro* and *in vivo*. As different AAV serotypes transduce cells with varying efficiencies, we have switched to an AAV1 vector. We have additionally used several different promoter elements, including CMV and most recently the CAG promoter for constitutive expression. Enhancer elements on the 3' end of the decorin transcript have also been deleted, resulting in a smaller amount of DNA packaged into the virus (in the AAV system, the size of packaged transgene is very important for efficient expression). These changes have resulted in the first positive in vitro gene transfer results (as assayed by Northern blot) we have seen with the AAV system. Further fine-tuning and changes should result in improved AAV transduction efficiency. Screening for decorin protein production via ELISA or Western will also be used.

Liposome-mediated gene transfer

Liposome-mediated gene transfer with the DC-Chol liposome system has shown no expression *in vitro* or *in vivo*. The large amounts of plasmid needed for the gene transfers (milligram range for each treatment), and the large working volumes needed made this system less feasible for *in vivo* tumor treatment, which is our ultimate goal. Other liposome systems are possible, as are new formulations of this system. Additionally, better decorin constructs with improved expression characteristics can be utilized. The use of 3' RNA stabilization elements could possibly enhance expression, as could the use of better a better signal peptide for the decorin protein to enhance secretion (we are using either the native SP or the insulin SP in our constructs).

Other gene transfer methods

Recent work in our lab, published in *Oncogene* and included in the Appendix, has shown a direct downregulation of EGFR phosphorylation after exposure of cells to a replication-incompetent adenoviral viral vector containing the human decorin transgene (Ad-Dcn). Additionally, cells dependent of the EGFR receptor pathway for stimulation and growth have been suppressed *in vitro* and *in vivo* by administration of this viral

vector (details are in the Appendix). The Ad-Dcn system has proven efficacious and useful both *in vitro* and *in vivo*. The adenoviral system is currently being tested in pilot gene transfer studies with mammary tumor xenografts in mice.

Investigation of mammary tumor cell lines
We have studied in vitro several different mammary tumor cell lines for the ability to produce consisten tumors in nu/nu and/or SCID mice. We are currently assessing viral transducibility, tumorigenicity in mouse models, growth rates, and decorin-mediated growth inhibition of these cell lines.

Inducible promoter system for decorin expression
We are screening several clones from several mammary tumor cell lines, as well as other cell lines overexpressing EGFR, for positive clones that can express the decorin gene under the control of an inducible promoter.

KEY RESEARCH ACCOMPLISHMENTS

- *in vitro* testing of several AAV viral vectors
- *in vitro* testing of adenoviral vector
- growth inhibition and downregulation of several cell lines observed after administration of adenoviral vector containing decorin
- *in vivo* administration of adenoviral vector to successfully treat tumor xenografts
- *in vitro* testing on adenoviral vector on breast carcinoma cell lines
- finding of breast carcinoma cell lines capable of forming tumors in *nu/nu* and/or SCID mice
- pilot studies on adenoviral vector to treat breast carcinomas in a xenograft tumor model
- screening of clones for inducible promoter to control decorin expression in breast carcinoma cells

REPORTABLE OUTCOMES

We have recently published an article in Oncogene, as well as presented results in poster form at the Proteoglycans Gordon Conference in Andover, New Hampshire.

- Reed, C.C., Gauldie, J., and Iozzo, R.V. (2002) Suppression of tumorigenicity by adenovirus-mediated gene transfer of decorin, Oncogene 21:3688-3695.

- Reed, C. C., Gauldie, J., and Iozzo, R. V. (2002) Suppression of tumorigenicity by adenovirus-mediated gene transfer of decorin. Gordon Research Conference, Andover, NH.

CONCLUSIONS

We are at an important juncture of our research- assessing the quality and feasibility of several different gene transfer methodologies as well as the ability of several tumor cell lines to generate xenograft tumors consistently and reproducibly. While the adenoviral system has shown a great deal of promise, it has the problem of increased immunogenicity as well as transient transgene expression. The AAV system, on the other hand, expresses for much longer term, up to and including years when it functions optimally (Samulski et al., 1999; During 1997). AAV's lesser immunogenicity and its ability to transduce cells long-term continue to make it a vector worth pursuing. Other work done in our lab has also raised interesting possibilities for future directions- we have recently defined the exact regions on the proteins where decorin and EGFR interact

(Santra et al, 2002). With this information, decorin mutations with improved binding characteristics can be designed intelligently. Peptide or peptidomimetic EGFR inhibitors based on the decorin region known to interact directly with EGFR are another future direction this therapy could take. We are exploring several novel inducible promoter systems, including one based on the HSP70 promoter (Romano et al, 2001). The HSP70 inducible promoter has the advantage of easy clonal selection as the inducible transgene is inserted by a retroviral delivery method and should be of high efficiency.

The ultimate goal of this research is to prove that human decorin is a viable candidate or adjunctive candidate for treatment of certain breast cancers, and that it is possible to deliver the decorin transgene by one or more well-established transfer methods to achieve appositive clinical response. Gene therapy strategies are being dev eloped and used because the continual treatment of diseases with actual protein runs into problems of long-term administration, as well as, at times, production and purification of the protein itself. Gene therapy seeks to avoid these pitfalls by delivering to local cells the ability to make the protein themselves, and to make it long-term, resulting in therapeutic effect. The associated problems of gene transfer are currently being investigated by not only our group but by many groups all over the world. If we can definitively prove decorin's efficacy, there will be soon, if not already, other improved gene delivery mechanisms to use for treatment of breast (and other) carcinomas that overexpress ErbB family receptors.

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Romano, G., Reiss, K., Tu, X., Peruzzi, F., Belletti, B., Wang, J.Y., Zanocco-Marani, T., and Baserga, R. (2001) Efficient in vitro and in vivo gene regulation of a retrovirally delivered pro-apoptotic factor under the control of the Drosophila HSP70 promoter. Gene Therapy, 8:600-607.

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APPENDICES

Attached is a copy of the *Oncogene* article recently published.

Oncogene (2002) 21, 3688–3695

www.nature.com/onc

Suppression of tumorigenicity by adenovirus-mediated gene transfer of decorin

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There is mounting evidence that decorin inhibits the growth of various tumor cell lines when either overexpressed *in vitro* or provided as a recombinant protein. The mechanism of action is primarily via a protracted inactivation of the epidermal growth factor receptor (EGFR) tyrosine kinase. In this study we explored the possibility of retarding the growth of tumor xenografts by decorin gene delivery into the growing neoplastic tissues. We demonstrate that transient transgene expression of replication-deficient adenovirus-containing decorin causes a significant growth inhibition of colon and squamous carcinoma tumor xenografts. These cytostatic effects were achieved with relatively low viral titers and correlated with a reduced proliferative index and an attenuation of the EGFR phosphorylation *in vivo*. Thus, decorin gene therapy helps in retarding the growth of human tumors in immunocompromised animals and could represent a new independent or adjunctive therapeutic modality against cancer.
Oncogene (2002) **21,** 3688–3695. DOI: 10.1038/sj/onc/1205470

Keywords: decorin proteoglycan; adenovirus gene therapy; colon carcinoma; squamous carcinoma

Introduction

Decorin is a prototype member of an expanding family of small leucine-rich proteoglycans, ubiquitous molecules characterized by 10–12 tandem arrays of leucine-rich repeats (Iozzo, 1997, 1998). The decorin protein core folds into an arch-shaped structure capable of interacting with various extracellular matrix proteins, growth factors and their receptors (Weber *et al.*, 1996). Targeted deletion of the decorin gene (Danielson *et al.*, 1997) and bitransgenic animals with deficiency of both decorin and p53 (Iozzo *et al.*, 1999a) have provided genetic evidence for a role of decorin in matrix assembly and tumorigenesis, respectively. The results of these studies have confirmed and corroborated earlier observations implicating decorin protein core in modulating collagen fibrillogenesis (Vogel *et al.*, 1984) and cell growth via a block of TGF-β^1 activity (Yamaguchi *et al.*, 1990).

Our working hypothesis is that decorin represents a natural antagonist of tumor growth. This hypothesis is based on several key observations that have shown a marked elevation of decorin expression within the tumor stroma of colon cancer (Adany *et al.*, 1990), an increased decorin gene transcription in serum-starved and quiescent cells (Coppock *et al.*, 1993; Mauviel *et al.*, 1995), and a marked suppression of decorin gene expression in most viral- or spontaneously-transformed cells (Coppock *et al.*, 1993; Adany *et al.*, 1990). Moreover, decorin expression is differentially down-regulated in hepatocellular carcinomas (Miyasaka *et al.*, 2001) and in ~70% of ovarian carcinomas and ovarian cancer cells (Shridhar *et al.*, 2001) as compared to their normal counterparts.

The mechanism of action of decorin has only recently begun to be elucidated by the discovery that decorin is a novel biological ligand of the EGFR (Moscatello *et al.*, 1998; Patel *et al.*, 1998; Iozzo *et al.*, 1999b). This interaction with the EGFR differs from that induced by EGF, since decorin leads to a protracted down-regulation of EGFR tyrosine kinase (Csordás *et al.*, 2000), and other members of the ErbB family of receptor tyrosine kinase (Santra *et al.*, 2000), and causes an attenuation of the EGFR-mediated mobilization of intracellular calcium (Csordás *et al.*, 2000). The decorin/EGFR interaction induces the expression of the endogenous cyclin-dependent kinase inhibitor $p21^{WAF1}$ (De Luca *et al.*, 1996) and a subsequent arrest of the cells in the G1 phase of the cell cycle (Santra *et al.*, 1995). These cytostatic effects of decorin occur in a wide variety of tumor cell lines with a diverse histogenetic background (Santra *et al.*, 1997; Nash *et al.*, 1999) and can also affect murine tumor cells (Santra *et al.*, 1997) and normal cells such as endothelial cells (Schönherr *et al.*, 2001) and macrophages (Xaus *et al.*, 2001).

Because decorin is a highly soluble compound, and because the activity of decorin occurs in an autocrine and paracrine fashion, we sought to

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determine whether adenovirus-mediated gene delivery into solid tumors could affect the behavior of the tumor xenografts. We demonstrate that transient transgene expression of replication-deficient adenovirus-containing decorin causes a significant growth inhibition of colon carcinoma and squamous carcinoma tumor xenografts. The cytostatic effects of decorin correlated with a markedly reduced proliferative index and an attenuation of the EGFR phosphorylation *in vivo*. Thus, decorin gene therapy helps in retarding the growth of human tumors in immunocompromised animals and could represent a new independent or adjunctive therapeutic modality against cancer.

Results

Efficient transduction of various tumor cell lines with Ad-Dcn

To determine whether Ad-Dcn was capable of efficiently transducing WiDr and A431 tumor cells, we added increasing amounts of Ad-Dcn vector or empty vector, and determined decorin expression by Northern blotting. The results showed a significant expression of the virally-transduced decorin gene transcript, a single ~1.9 kb band (Figure 1a), whose levels increased in a dose-dependent manner. In agreement with previous results (Santra *et al.*, 1995; Moscatello *et al.*, 1998), no decorin message was detected in either control tumor cell (Figure 1a). Real time PCR showed that the expression of the decorin transcript was time-dependent in A431 cells (Figure 1b), and could also be demonstrated in Cos-7 and HeLa cells (Figure 1c). Moreover, following a 48 h-incubation, the message levels induced by Ad-Dcn were higher than those induced by a bacterial expression vector (pcDNA) harboring the potent CMV promoter (not shown). Western immunoblotting showed significant production of decorin proteoglycan (Figure 1d), a ~100-kDa broad band, with a migration similar to that described before for stable expressing clones of WiDr and A431 (Santra *et al.*, 1995, 2000), and typical of recombinant decorin proteoglycan (Hocking *et al.*, 1998; Ramamurthy *et al.*, 1996; Mann *et al.*, 1990). To further confirm the biological activity of decorin, we reacted Ad-Dcn-transduced cells with a monoclonal antibody (mAb E12120) that specifically recognizes the phosphorylated (activated) form of the EGFR. The results showed a marked suppression of the EGFR phosphorylation in the A431 cells when compared to either control (no virus) or the empty viral vector (Figure 1e). Thus, our Ad-Dcn vector is capable of inducing high levels of decorin expression that can be detected at least 10 days after transduction in several cell lines. Moreover, decorin is biologically active since it can down-regulate the EGFR kinase as in stably transfected tumor cell lines (Csordás *et al.*, 2000; Santra *et al.*, 2000).

Suppression of tumor growth by direct intra-neoplastic injection of Ad-Dcn

The high efficiency of transduction and the relatively high levels of transgene expression mediated by the adenoviral vector in cultured cells prompted us to investigate the anti-oncogenic properties of decorin *in vivo* by direct intra-neoplastic injection of the Ad-Dcn. To this end, we performed two sets of experiments. In the first set of experiments, animals were injected s.c. with a relatively large inoculum of tumor cells (2×10^6 cells/animal), and the mice were observed for 5–7 days until a palpable tumor of ~30 mm^3 emerged. At this time, each tumor xenograft was directly injected with 50 μl of PBS containing 7×10^7 pfu of either Ad-Dcn or empty viral vector. A second treatment was done 4–5 days later. Interestingly, in both WiDr (Figure 2a) and A431 (Figure 2b) tumor xenografts there was a significant reduction of tumor growth ($P<0.02$). However, the effect was rather transient since the slope of the curve was nearly identical in both treated and untreated animals at later times. In addition, because of the large inoculum size, the growth rate of both xenografts was very rapid and, thus, several tumors in both groups had necrotic centers, thereby preventing an accurate assessment of the decorin's effects.

In a second set of experiments we tried to duplicate a more clinically relevant situation by injecting smaller inocula, and by treating the preestablished tumor xenografts with higher doses and multiple injections of recombinant Ad-Dcn. The results showed a marked inhibition of tumor growth in both the colon carcinoma (Figure 2c) and squamous carcinoma (Figure 2d) xenograft models. In both cases, a progressive inhibition of tumor burden was observed. At the end of the experiments, the decrease in tumor volume for the Ad-Dcn-treated colon carcinoma xenografts reached 67% ($P<0.001$) (Figure 2c), and 52% inhibition ($P<0.002$) for the squamous carcinoma xenografts (Figure 2d). Thus, topical delivery of Ad-Dcn has substantial cytostatic effects in two animal models of aggressive human tumors.

Adenoviral-mediated expression of decorin causes morphological changes in tumor xenografts

We studied the morphology of the tumor xenografts generated with smaller inocula and multiple Ad-Dcn injections. While the wild-type WiDr showed solid tumors (Figure 3a) with invasion of the deep fascia and skeletal muscle (not shown), the Ad-Dcn-treated neoplasms showed well demarcated margins (Figure 3b) and often showed areas of glandular differentiation (Figure 3c). Similarly, the A431 squamous carcinoma xenografts showed invading margins (Figure 3d), whereas the Ad-Dcn-treated counterparts showed sharp, non-invading borders (Figure 3e), and areas of squamous differentiation characterized by the formation of numerous keratin pearls (Figure 3f). Thus, the decorin-induced growth inhibition is also associated

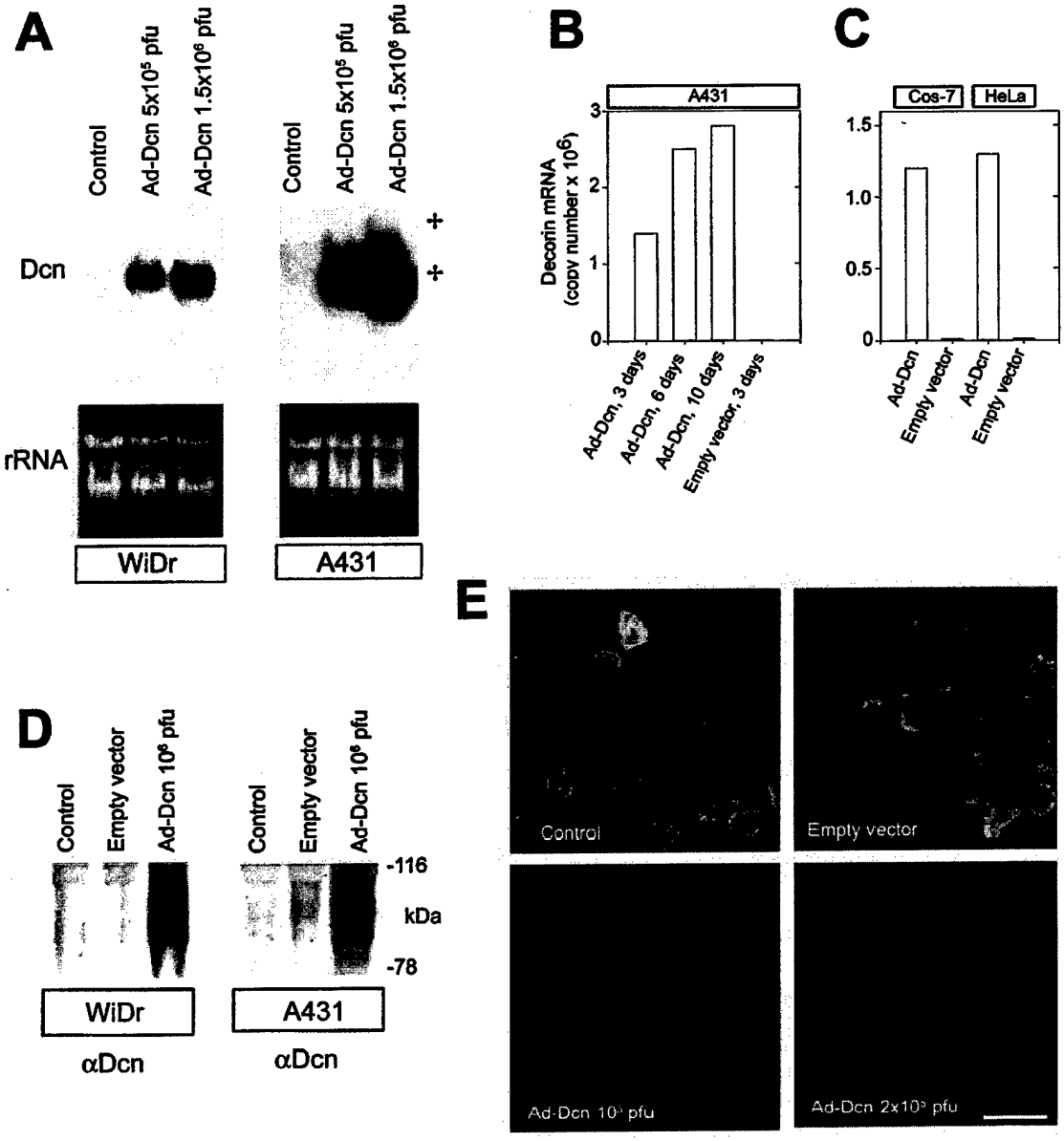

Figure 1 Efficient transduction of various tumor cell lines with Ad-Dcn. (**a**) Northern blotting of total RNA extracted from the designated cells 2 days after transduction with either 2.5×10^6 pfu of empty viral vector (Control) or with increasing amounts of Ad-Dcn as indicated in the top margin. The autoradiographs were exposed for 2 h. The asterisks in the top right panel correspond to the migration position of the two ribosomal RNAs. (**b**) Real time PCR values of decorin gene expression 2 days following transduction with either Ad-Dcn or the empty viral vector (10^6 pfu each) for the indicated times. Values represent the mean of duplicate determinations and are based on a standard DNA curve generated with known quantities of human decorin full-length cDNA, as per manufacturer's protocol (Applied Biosystems 7700 ABI Prism Sequence Detector). (**c**) Transduction of monkey Cos-7 or human squamous carcinoma HeLa cells with either Ad-Dcn or empty viral vector as indicated. (**d**) Western immunoblotting of media conditioned for 48 h by WiDr or A431 carcinoma cells following transduction with Ad-Dcn viral vector as indicated. The precipitated media aliquots were reacted with an anti-decorin antibody (αDcn) directed against the N-terminal region (Fisher *et al.*, 1995). The migration of pre-stained molecular weight markers is shown in the right margin. (**e**) Immunofluorescence staining of A431 squamous carcinoma cells following transduction for 72 h with Ad-Dcn at the designated concentrations, control (no virus) or empty vector, using a monoclonal antibody directed against the phosphorylated form of the EGFR (BD Transduction Laboratories). Cells were fixed for 15 min in 3% paraformaldehyde, permeabilized for 10 min with digitonin, and incubated with the primary antibody (1 : 500). Slides were washed and incubated with secondary goat anti-mouse IgG-FITC (1 : 1000) antibody. Immunofluorescence was performed on an Olympus IX70 inverted epifluorescence microscope coupled to a high quantum efficiency cooled CCD camera. The four panels were exposed for the same amount of time. Notice the significant reduction in EGFR signal in both decorin-transduced cells. Scale bar = 50 μm

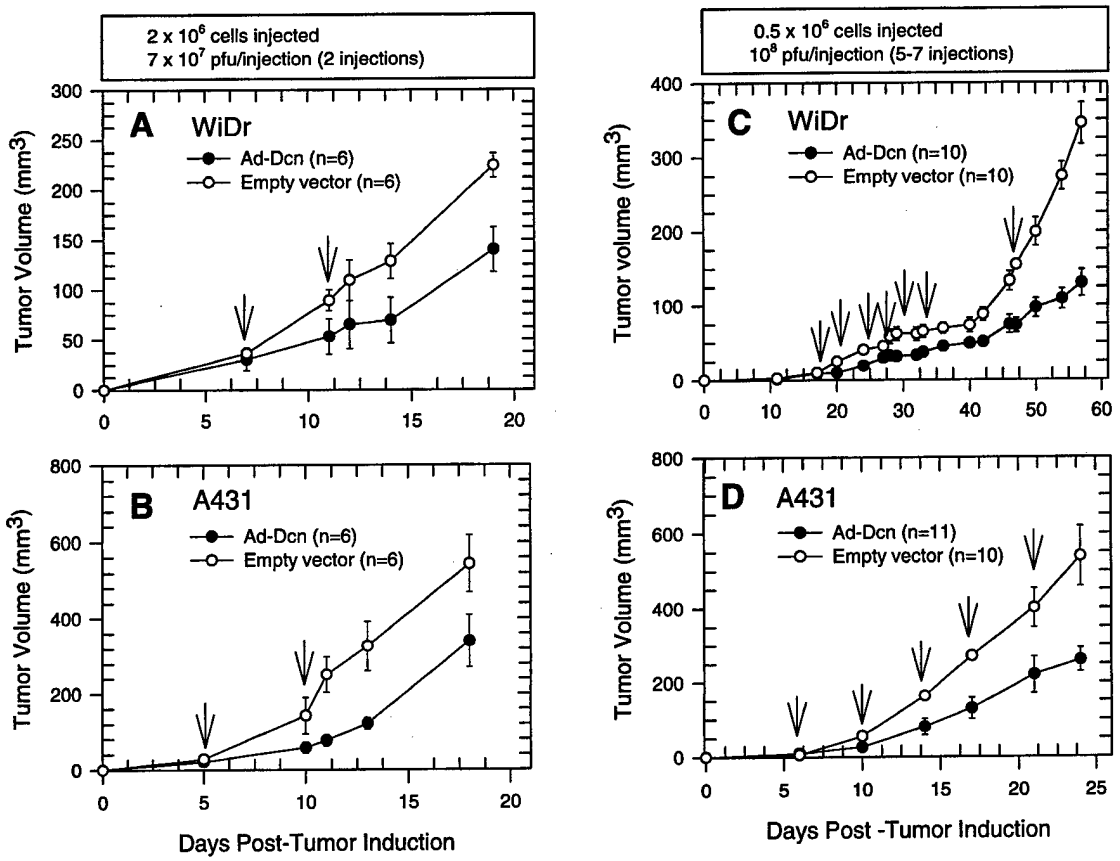

Figure 2 Intra-neoplastic injections of Ad-Dcn reduce the growth of preestablished tumor xenografts. (**a** and **b**) About 2×10^6 WiDr or A431 cells were injected s.c. into the dorsal region of *nu/nu* mice ($n=6$ for each group). At the indicated time points (vertical arrows) the tumors were directly injected with 50 μl of PBS containing 7×10^7 pfu of either recombinant Ad-Dcn or empty viral vector, as indicated. The values represent the mean$\pm$s.e.m. (**c** and **d**). In these experiments a smaller inoculum of $\sim0.5\times10^6$ cells was injected and several, higher-titer injections were performed at the designated times. The values represent the mean$\pm$s.e.m. of 10–11 animals per group as indicated

with changes in the architecture and differentiation of the tumor xenografts.

Decorin gene expression correlates with a decreased proliferative index and an attenuation of EGFR activation

Next, we sought to determine whether decorin gene transfer would correlate with a reduced proliferative index and a reduced activation of the EGFR. To test these possibilities we stained tumor samples with antibodies directed against Ki-67, an established proliferation marker, and a monoclonal antibody (mAb E12120) that specifically recognizes the phosphorylated (activated) form of the EGFR (see above). There was a marked reduction of the Ki-67 staining in the treated tumor xenografts (Figure 4b,d) as compared to the vector controls (Figure 4a,c). To obtain the proliferative index, we estimated the percentage of tumor cell nuclei positive for Ki-67 marker in 10 high-power ($\times400$) fields per animal ($n=3$ animals per group). The proliferative index for the WiDr and A431 controls was $36\pm6\%$ and $28\pm5\%$, respectively (mean$\pm$s.e.m.). In contrast, in the Ad-Dcn-treated samples, the Ki-67 proliferative index was $6\pm2\%$ and $5\pm3\%$, respectively ($P<0.001$ in both cases).

The staining for the activated EGFR was also diminished in the treated samples (Figure 4f,h) when compared to vector controls (Figure 4e,g). In the WiDr xenografts there was a linear staining outlining the cellular contours (Figure 4e). In agreement with their high content of EGFR, A431 cells reacted more intensely with the anti-EGFR antibody. Figure 4g shows a pocket of intensely-stained A431 tumor cells just below the unreactive mouse epidermis. These data are also in agreement with the *in vitro* data shown in Figure 1e.

When the tumor xenografts were reacted with a polyclonal antibody specific for the N-terminal peptide of the human decorin protein core, we detected decorin epitopes in pericellular pools mostly located near the periphery of the tumor xenografts (Figure 4j,l), while the vector controls were unreactive (Figure 4i,k).

Collectively, these results provide strong evidence for the *in vivo* anti-proliferative effects of decorin and suggest that, at least in part, decorin inhibits the proliferation of tumor cells by interfering with EGFR activity.

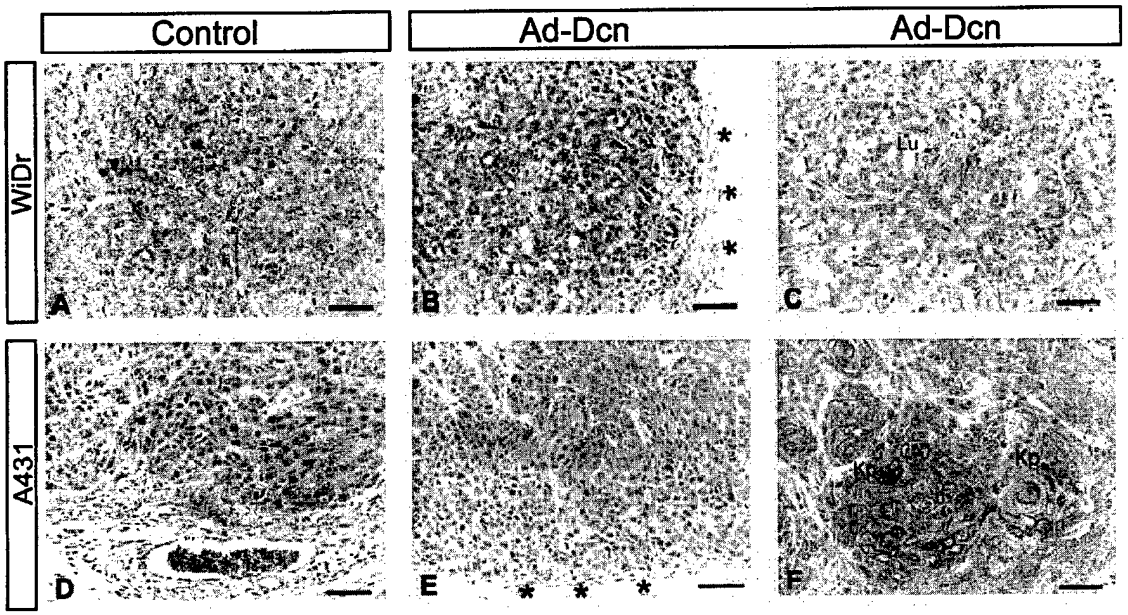

Figure 3 Adenoviral-mediated expression of human decorin causes morphological changes in human tumor xenografts. Gallery of light micrographs highlighting the morphology of the tumor xenografts generated by injection of WiDr colon carcinoma cells (**a**–**c**) or A431 squamous carcinoma cells (**d**–**f**) into the dorsal subcutaneous tissue of *nu/nu* mice. These sections are from the animals in Figure 2c,d and show representative fields of tumor xenografts of mice treated with multiple injections of either empty adenoviral vector (Control) or Ad-Dcn as indicated. Asterisks in **b** and **e** point to the sharper margins of the Ad-Dcn-treated tumor xenografts; *Lu*, lumen; Kp, keratin pearl. Scale bars = 125μm

Discussion

The inhibitory effects of decorin on TGF-β activity have been exploited in experimental glomerulonephritis (Isaka *et al*., 1996), atherosclerosis (Fischer *et al*., 2001), lung morphogenesis (Zhao *et al*., 1999), and pulmonary fibrosis (Kolb *et al*., 2001). This is the first report of a successful *in vivo* treatment of two human xenograft models using adenovirus-mediated decorin gene transfer. The results showed a marked inhibition of tumor growth in both the colon and squamous carcinoma xenograft models. In both cases, a progressive inhibition of tumor burden was observed. In Ad-Dcn-treated colon carcinoma xenografts this reduction was greater than squamous carcinoma xenografts (67% versus 52% of control values) but in both cases the difference was statistically significant with P values <0.001 and <0.002, respectively). We should point out that the total amount of Ad-Dcn injected is still below what has been used in most animal experiments and recent clinical trials. In fact, the highest total dose/animal (see Figure 2c) reached 7×10^8 pfu/22 g (the average weight of a *nu/nu* mouse), or equivalent to a dose of $\sim3.2\times10^{10}$ pfu/kg in a human. Thus, topical delivery of Ad-Dcn has a significant cytostatic effect in two animal models of aggressive human tumors.

Interestingly, adenoviral-mediated expression of decorin caused morphological changes in the human tumor xenografts. These changes included sharper border, reduced invasiveness, reduced angiogenesis and evidence of cyto-differentiation. Whether these changes are the direct results of decorin treatment or a consequence of reduced tumor growth is not clear, but it warrants future investigation. The morphologic appearance of the transduced tumors is reminiscent of that obtained with xenografts produced by co-injection of wild-type and decorin-expressing A431 cells (Csordás *et al*., 2000). In the latter case, tumors grew only when the ratio of wild-type to decorin-expressing cells was quite high ($\geqslant1:2$), and in the tumors generated at lower (1 : 4 or 1 : 8) ratios there was evidence of cyto-differentiation and sharper, non-invasive tumor boundaries. Thus, the Ad-Dcn vector can produce effects similar to those induced by a constant source of decorin derived from the stably expressing tumor cell clones (Santra *et al*., 2000). The present study extends these observations and supports the feasibility of decorin gene transfer in a clinically relevant setting, either as primary or combined therapy. The latter possibility is supported by the synergistic action of decorin and carboplatin in the *in vitro* inhibition of human ovarian cancer cells (Nash *et al*., 1999).

Human carcinomas frequently express high levels of EGFR and over-expression of EGFR and ErbB2 has been correlated with a more aggressive phenotype (Carpenter, 2000). Accordingly, therapeutic modalities directed at preventing the function of these receptors have been pursued as useful anti-cancer treatments (Mendelsohn and Baselga, 2000). A key finding of our study is that decorin gene expression in the treated tumor xenografts correlated well with a decreased proliferative index and an attenuation of EGFR activation. The latter observation is a novel and

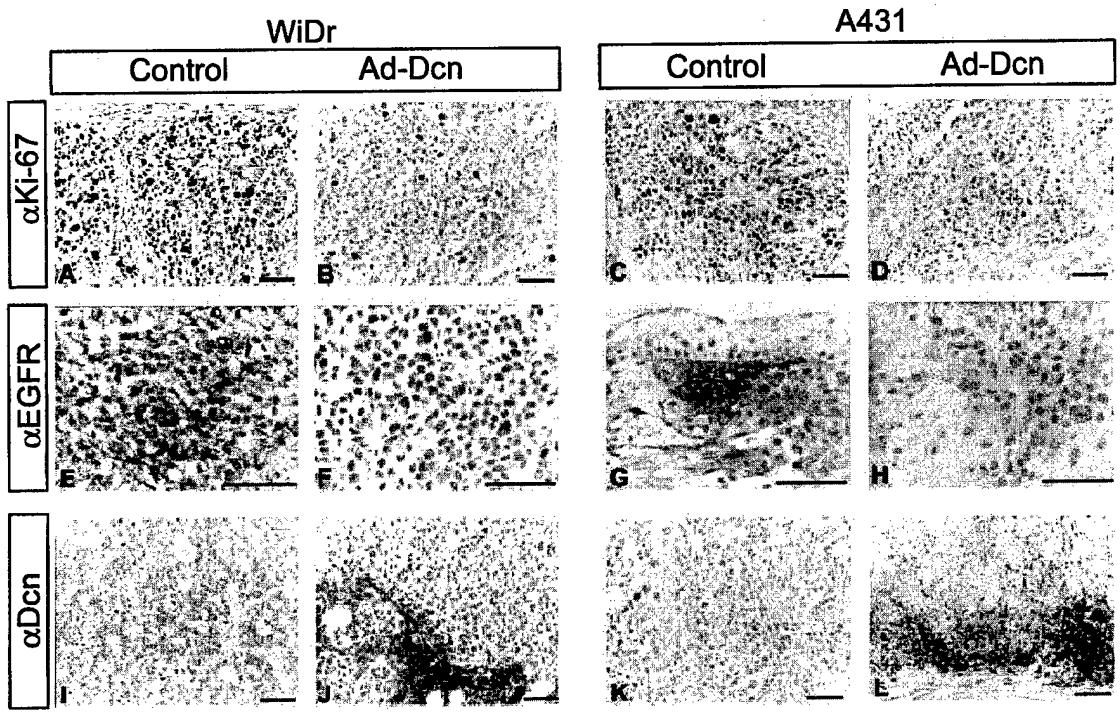

Figure 4 Expression of Ki-67, activated EGFR and decorin in WiDr colon carcinoma and A431 squamous carcinoma xenografts treated with either empty adenoviral vector (Control) or Ad-Dcn. (**a**–**d**) Representative photomicrographs of sections stained with a mouse monoclonal antibody (B126.1) against the nuclear proliferation marker Ki-67 (Biomeda Corp.), used at 1 : 100 dilution. Notice the markedly reduced number of cells with positive nuclear staining in the Ad-Dcn treated samples. (**e**–**h**) Representative photomicrographs of sections stained with a mouse monoclonal antibody (E12120) raised against the activated (phosphorylated form) of the human EGFR (Transduction Laboratories), used at 1 : 500 dilution. Notice the linear staining outlining the contours of WiDr cells (**e**) and the markedly diminished staining in the Ad-Dcn-treated xenograft (**f**). In A431 xenografts there is a more intense staining because of the large number of EGFRs ($1-2\times10^6$ receptors/cell) expressed by these neoplasic cells. Notice the presence of a strongly reactive group of tumor cells just below the unreactive mouse epidermis (**g**) and the diminished reactivity in the Ad-Dcn treated tumor xenograft. (**h**). (**i**–**l**), representative photomicrographs of sections stained with a rabbit affinity-purified antiserum raised against the N-terminal peptide of human decorin (Fisher *et al.*, 1995), used at 1 : 500 dilution. Notice the mostly perpendicular deposits of decorin-containing epitopes only in the Ad-Dcn treated xenografts (**j** and **l**). Scale bars = 140 μm

important finding since this is the first report that decorin gene delivery alters EGFR activity *in vivo*. There is strong genetic evidence to support this important pathway of EGFR inactivation (Moghal and Sternberg, 1999; Carraway and Sweeney, 2001). In *Drosophila melanogaster*, for instance, there is a protein, named Kekkon1 (Musacchio and Perrimon, 1996), that acts in a feedback loop to negatively regulate the *Drosophila* EGFR during oogenesis (Ghiglione *et al.*, 1999). Kekkon1 contains six leucine-rich repeats homologous to decorin and accumulates in the dorsal-anterior follicle cells in an EGFR-dependent manner (Ghiglione *et al.*, 1999; Musacchio and Perrimon, 1996). Notably, loss and gain of Kekkon1 augments or suppresses EGFR activity, respectively, and the two proteins interact physically when co-expressed in cells (Ghiglione *et al.*, 1999). These findings suggest a model where Kekkon1 may interfere with ligand activation or may facilitate the negative regulation of EGFR following activation (Carraway and Sweeney, 2001). It is likely that mammalian cells may also utilize similar leucine-rich structures to counteract the EGFR. In contrast to Kekkon1, which is a trans-membrane protein and, thus, able to interact only with EGFR molecules on the same or adjacent cells, the soluble decorin could diffuse distantly and potentially interact with numerous target cells expressing varying levels of EGFR.

While adenovirus vectors induce immuno-reactivity in humans, thereby limiting their multiple use, other gene delivery systems, such as adenovirus associated viral vectors, are being rapidly developed. Thus, we believe that the safe and repeatable gene transfer of decorin-containing viral vectors could represent a novel therapeutic tool against cancer. There are a number of advantages of using decorin as a primary or adjuvant therapy. Decorin, being a secreted proteoglycan, could interact with a number of cell-surface receptors at distant sites of transduction (Iozzo, 1999). Conceivably, a single decorin-transduced cell could affect several neighboring cells, thereby rendering this therapeutic modality effective even in systems with low or unpredictable transduction efficiency. An increase in decorin content in the pericellular environment of solid tumors, such as colon and squamous carcinomas, could trigger a functional interaction with the EGFR and other members of the ErbB family of receptor tyrosine kinase, thereby initiating a signaling cascade

that would lead to growth retardation or arrest. Decorin is a natural product and, therefore, there should not be any immune response to it. Moreover, over-expression of decorin has profound cytostatic effects preferentially on malignant cells independent of their histogenetic origin (Santra *et al.*, 1997). At least *in vitro*, smooth muscle cells appear to react differently to decorin, primarily by responding to the decorin-mediated block of TGF-β activity (Fischer *et al.*, 2001), while in macrophages recombinant decorin can enhance cell survival (Xaus *et al.*, 2001). Thus, although the pleiotropic effects of decorin need to be elucidated in future studies, our results provide support to the hypothesis that decorin could be used as a therapeutic modality to combat cancer, either as a primary or an adjuvant compound. The lack of toxicity, its solubility and its mode of action, i.e. an action on multiple cells and at a distance, make decorin gene therapy an attractive mode of intervention for numerous forms of human malignancy.

Materials and methods

Cell lines, antibodies and adenovirus production

Human WiDr colon carcinoma, A431 squamous carcinoma cells, 293 embryonic kidney cells, HeLa squamous cell carcinoma and monkey Cos-7 cells were obtained from the American Type Culture Collection. The following antibodies were used: a monoclonal antibody (E12120) raised against the activated (phosphorylated form) of the human EGFR (BD Transduction Laboratories); a rabbit affinity-purified antiserum raised against the N-terminal peptide of human decorin (Fisher *et al.*, 1995); a mouse monoclonal antibody (B126.1) against the nuclear proliferation marker Ki-67 (Biomeda Corp., Foster City, CA, USA). Full-length human decorin cDNA was cloned into a shuttle vector containing the human CMV promoter and the E1 region of the human adenovirus type 5 genome. The recombinant adenovirus-decorin (Ad-Dcn) was generated by homologous recombination after co-transfection with the shuttle vector and a virus-rescuing vector as described before (Zhao *et al.*, 1999). Samples of both decorin and control vectors were step-amplified in increasing sizes of low passage 293 cells, using successive rounds of freezing and thawing, and centrifugation. High titers of Ad-Dcn or empty vector were purified by CsCl density gradient ultracentrifugation and chromatography on pre-packed G-25 columns. Purified viruses were plaque titered in 293 cells and expressed as plaque-forming units (pfu). Alternatively, virus was purified with the Viraprep Kit (Virapur, Carlsbad, CA, USA), and viral titer was determined by $TCID_{50}$ assay.

Viral transduction, Northern blotting, real time PCR and immunofluorescence

WiDr and A431 cells were cultured in a 6-well tray. While sub-confluent, Ad-Dcn (5×10^5, and 1×10^6 pfu) was added to the media. Cells were grown for 2–10 days without media change before harvesting for RNA. In addition, Cos-7 and HeLa squamous carcinoma cells were transduced. As a further control, human decorin cDNA in pcDNA3 plasmid (Invitrogen, Carlsbad, CA, USA) was transfected in cells using Transit LT-1 (PanVera, Madison, WI, USA), Cells were drained of media and RNA was extracted with Tri Reagent (Sigma, St. Louis, MO, USA). RNA was run on a 1% agarose gel with 10% formaldehyde in 1× MOPS and stained with ethidium bromide for photography. RNA was transferred onto a nylon membrane (Osmonics, Westborough, MA, USA) and cross-linked with UV Strata linker (Stratagene, Cedar Creek, TX, USA). A human decorin cDNA probe was produced by PCR of full-length human decorin cDNA. Forward primer AAGAACCTTCACG-CATTG and reverse primer TCCAACTACAGAGATATT were used to generate a 611 bp fragment which was labeled with ^{32}P-dCTP using the Random Primer DNA Label System (Life Technologies, Rockville, MD, USA). Blots were hybridized with a radio-labeled probe in 1 M NaH_2PO_4 at pH 7.2, 1 mM EDTA, 7% SDS, and 1% BSA overnight, thoroughly washed, and exposed to film for 2–4 h at room temperature. Reverse transcription reaction was performed on RNA with SuperScript II Reverse Transcriptase (Life Technologies, Rockville, MD, USA). Real time PCR was carried out on samples as per manufacturer's protocols (Applied Biosystems 7700 ABI Prism Sequence Detector) using forward primer AGTGACTTCTGCCCACCCTGG and reverse primer CCGGGTTGCTGAAAAGACTC. Quantization of message was based on standard DNA curve generated with known quantities of human decorin cDNA. For immunofluorescence, cells were cultured in chamber slides (Nunc, Napervile, IL, USA), and, while sub-confluent, they were transduced with adenoviral decorin (1×10^5 pfu and 2×10^5 pfu) or control vector (2×10^5 pfu) for 72 h. Following a 15 min fixation in 3% paraformaldehyde, the cells were permeabilized for 10 min with digitonin (30 μg/ml), blocked with 1% BSA for 1 h, and incubated for 30 min with the primary antibody (1 : 500) against the phosphorylated (activated) form of the human EGFR (BD Transduction Laboratories, Franklin Lakes, NJ, USA). Slides were washed with 1% BSA and incubated with secondary goat anti-mouse IgG-FITC antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at a 1 : 1000 dilution for 30 min. Immunofluorescence microscopy was performed on an Olympus IX70 inverted epifluorescence microscope coupled to a high quantum efficiency cooled CCD camera. Digital images were imported into Adobe® Photoshop 6.0.

Animal experimemts, morphology and immunohistochemistry

Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23) and the Institutional Animal Care and Use Committee of Thomas Jefferson University. Human colon and squamous cell carcinoma xenografts were established in athymic nude *nu/nu* mice, 6–8 weeks of age (Charles River Laboratories) through subcutaneous inoculation of $0.5-2\times10^6$ cells into the dorsal flank of each mouse. Mice were carefully examined every 2 or 3 days and any tumor growth was measured with a micro-caliper according to the following formula: $V=a\,(b^2/2)$, where a and b represent the larger and smaller diameters, respectively. When tumors had reached 2–3 mm in greater diameter, each mouse received direct intra-neoplastic injections ($\sim$50 μl containing $0.7-1\times10^8$ pfu) of replication-incompetent adenovirus, either empty virus or virus harboring the full-length decorin gene. Student's two-sided t-test was used to compare the values of the treated and control samples. A value of $P<0.05$ was considered as significant.

Animals were sacrificed at the end of the experiments, between 19 and 58 days depending on the treatment regimen and inoculum size, and each tumor was carefully dissected. The tumors were fixed in 10% buffered formaldehyde, embedded in paraffin and processed for routine histology. For immunohistochemistry, sections placed on poly-L-lysine-coated glass slides were de-paraffinized, and epitope retrieval was performed in 10 mM EDTA buffer for 10 min in a microwave at 600 W, or by incubating the slides with pepsin for 10 min. After epitope retrieval, endogenous peroxidase was blocked in 0.03% H_2O_2, washed in PBS and incubated with the primary antibodies diluted between 1:100 and 1:500. The peroxidase-labeled polymer conjugated to either goat anti-rabbit or anti-mouse method (DAKO EnVision+ System, DAKO Corp., Carpinteria, CA, USA) was used to detect antigen-antibody reaction. Specific reactions were visualized with 3,3'-diaminobenzidine as a chromogen and images were captured with a Pixera digital camera and processed using Adobe® Photoshop 6.0. To determine the proliferative index of tumor xenografts, we estimated the percentage of tumor cell nuclei positive for Ki-67 marker in 10 high-power (×400) fields per animal ($n=3$ animals per group). Additional details are provided in the legends to figures.

Abbreviations

Ad-Dcn, adenovirus-decorin; EGFR, epidermal growth factor receptor; TGF-β, transforming growth factor-β; pfu, plaque-forming units.

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